EFFECTS OF LIGHT ON CAROTENOGENESIS AND SPORULATION IN THE FUNGUS VERTICILLIUM AGARICINUM

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BY

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Abstract

Effects of light on carotenogenesis and sporulation as well as the subcellular distribution of carotenoids were investigated in the fungus Verticillium agaricinum (Link) Corda.

The action spectrum for carotenogenesis, obtained by exposing mycelial pads to monochromatic radiation, has maxima at 290 (main peak) and 390 nm, and a minor peak at 483 nm. An interaction between broad band ultraviolet (UV) (maximum at 313 nm) and blue light (maximum at 450 nm) was detected. Blue light partly reversed the UV induction of carotenogenesis when given after UV, but had no effect when given before UV. Light-induced absorbance changes found in this fungus were due to photobleaching of a pigment with absorption maximum at about 380 nm. The action spectrum of this bleaching also has a maximum at 380 nm and the bleaching does not seem to have any connection with carotenogenesis.

In 3-day-old colonies developed from the center of plates, UV or blue light was necessary for stimulating sporulation. However, when growth was started by inoculating with conidial suspension spread evenly over the plate, sporulation occurred readily in darkness, and blue light was found to inhibit sporulation. The inhibitory effect of blue light could be reversed by UV, but further effect of blue light was lost. The inhibition by blue light was also prevented by UV administered before the blue lgiht.

After disruption of the mycelia and upon further fractionation two major kinds of carotenoids were found. They are torulene (90%), found in the lipid layer on top of the supernatant, and an unidentified carotenoid (10%) associated with both mitochondria and endoplasmic reticulum. The carotenoid content was about 0.6 and 0.7-1.0 ug (mg protein)$^{-1}$ for mitochondria and endoplasmic reticulum, respectively.

Key words Verticillium agaricinum, fungi, conidiation, sporulation, UV and blue light, subcellular distribution of carotenoids, mitochondria, endoplasmic reticulum

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6 Acknowledgements

7 References

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1 List of papers

This thesis is based on the following papers, which will be referred to in the text by the Roman numerals I-V.

I Hsiao, K.-C. and Björn, L. O. 1982. Aspects of photoinduction of carotenogenesis in the fungus Verticillium agaricinum. - Physiol. Plant. 54: 235-238.

II Hsiao, K.-C. and Björn, L. O. 1982. Light-induced absorbance changes in the fungus Verticillium agaricinum. - Physiol. Plant 55: 73-75.

III Hsiao, K.-C. and Møller, I. M. 1984. The subcellular distribution of carotenoids in light-grown Verticillium agaricinum. - Physiol. Plant. (accepted)

IV Kumagai, T. and Hsiao, K.-C. 1983. Effect of light and phosphate on conidiation in the fungus Verticillium agaricinum. - Physiol. Plant 59: 248-252.

V Hsiao, K.-C. 1984. Sporulation in the fungus Verticillium agaricinum: Reversal of blue light inhibition by ultraviolet radiation. - Physiol. Plant. 60: 444-448.

2 General introduction

Developmental and physiological processes in living organisms are controlled not only by genetic information but also by the physical and chemical factors of the environment. One factor influencing development is light. Through evolution, living organisms have developed the ability to use light as a source of information about their immediate environment. This enables them to adopt the best strategy for survival. Information about the light environment can be processed to modulate a complexity of responses in fungi, for example, phototropism, phototaxis, pigment synthesis and sporulation. The last two types of photoresponse, namely carotenogenesis and sporulation, are the subjects of this study.

Carotenoid synthesis in a number of fungi is strictly under photocontrol (Rau 1976). While only trace amounts of carotenoids are produced by these fungi in the dark, a brief exposure to light results in substantial carotenoid synthesis. Studies of this phenomenon were conducted principally with Fusarium aquaeductuum, Neurospora crassa and Verticillium agaricinum. F. aquaeductuum has an action spectrum (Rau 1967) with maxima at 430-440 nm and 450-455 nm, a shoulder around 470-480 nm and a somewhat smaller band of activity at about 375 nm (Fig. 1a). This spectrum is very similar to

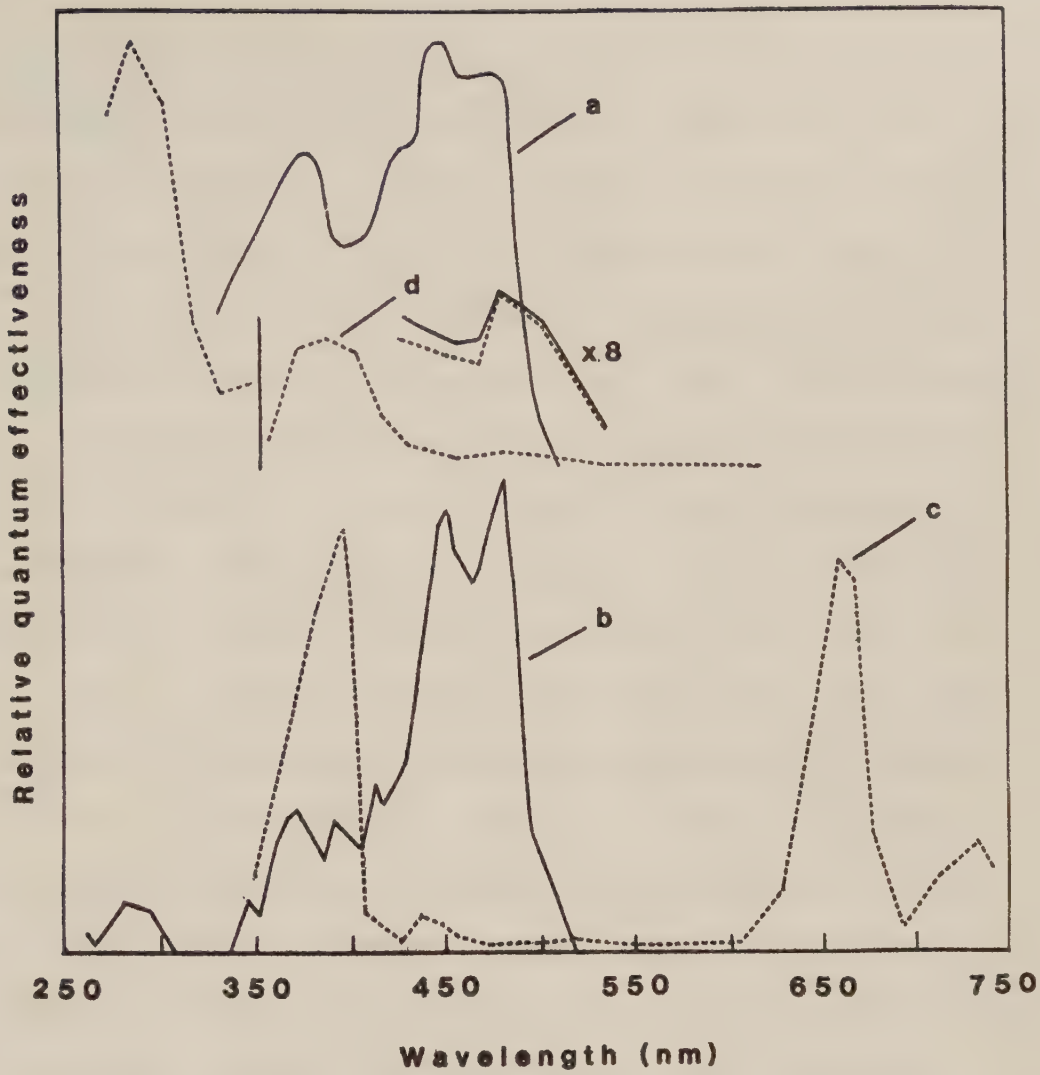

Fig. 1. Action spectra for carotenogenesis in fungi: (a) Fusarium aquaeductuum (Rau 1967); (b) Neurospora crassa (De Fabo et al. 1976); (c) Verticillium agaricinum (Valadon et al. 1982); (d) Verticillium agaricinum (I). The segment from 437 to 534 nm was determined anew, and was plotted with 8 times magnification of the vertical scale. Fluence rates were measured with a spectroradiometer (Optronic Laboratories, Inc., Model 742, Orlando, Florida) (dashed line) and a Lambda LI-188 quantum-radiometer-photometer (LI-COR, Ltd., Lincoln, Nebraska). Spectra were redrawn with permission.

the absorption spectrum of flavin, and therefore a flavoprotein has been suggested as the photoreceptor (Rau 1976). The action spectrum for N. crassa has been

determined by Zalokar (1955), and a more detailed one determined by De Fabo et al. (1976) is shown in Fig. 1b. The action maxima are at 481 and 450 nm in the visible range and two lesser peaks at 370 and 280 nm in the ultraviolet range. The response to light of wavelength between 350 and 380 nm is lower than one would expect from a process mediated by flavin, and therefore a carotenoid, rather than a flavin was proposed as photoreceptor. The action spectrum for V. agaricinum (Valadon et al. 1982) is quite different from those of N. crassa and F. aquae-ductuum, in that blue light was ineffective in inducing carotenogenesis and action maxima occurred at 395 and 650 nm (Fig. 1c). Apparently carotenogenesis in V. agaricinum is not mediated through typical blue light photoreceptors such as flavins or carotenoids. Our interest in V. agaricinum was initiated by a report which demonstrated the presence of phytochrome (Butler et al. 1959) by absorption difference spectroscopy and by red/far-red light reversible control of carotenogenesis (Valadon et al. 1979). We were, however, unable to confirm these results with our strain of V. agaricinum which turned out to be insensitive to red light (Tab. 2, I). It seems that our strain contains yet another different type of photoreceptor as indicated by the action spectrum for photocarotenogenesis (Fig. 1d).

Spectrophotometrical measurements of light-induced absorbance changes is a convenient method for detecting molecules undergoing a photochemical change or a

photochemical reaction catalyzed by the excited photoreceptor. A good example is the assay of the photochromic pigment, phytochrome (Butler et al. 1959) in which red/far-red reversible absorbance changes are recorded. Light-induced absorbance changes have been demonstrated in several fungi (Muñoz and Butler 1975, Lipson and Presti 1977). The absorbance changes showed the photoreduction of a b-type cytochrome(absorbance increase at 429, 520 and 555 nm) and photoreduction of a flavin (absorbance decrease at around 450 nm). Action spectra for these absorbance changes suggested a flavin functioning as photoreceptor. A light-induced absorbance decrease at 384 nm was detected in V. agaricinum. This wavelength was very close to the action maximum (390 nm) in the action spectrum for carotenogenesis (Fig. 1d), indicating a possible connection between the two events; this possibility was investigated (paper II).

One question that arose in connection with the study of carotenogenesis in the fungus was the intracellular location of carotenoids which has been studied only in a few species. The pigment has been reported to be present in the mitochondria (Neupert and Ludwig 1971, Ramadan-Talib and Prebble 1978) or mainly in endoplasmic reticulum (ER) (Mitzka-Schnabel and Rau 1980) of N. crassa. It is not known if the discrepancy is due to the differencein strain used in these investigations. Riley and Bramely (1976) suggested that carotenoid association with mitochondria or endoplasmic reticulum is a preparation artifact. To better understand the

physiological significance and biosynthetic mechanism of photocarotenogenesis in V. agaricinum, the subcellular distribution of this pigment was investigated (paper III).

Another interesting aspect of photomorphogenesis in fungi is the influence of light on the formation of reproductive structures like pycnidium and perithecium and conidia (Tan 1978). Light, especially near UV and blue light, stimulates sporulation in various fungi: Trichoderma viride (Gressel and Hartman 1968, Kumagai and Oda 1969), Aspergillus ornatus (Stallings 1970 as cited by Tan 1978) and Penicillium isariiforme (Bennink 1971 as cited by Tan 1978). Near UV and blue light can also have opposite effects; sporulation is promoted by near UV but inhibited by blue light. In a sequential irradiation with alternating near UV and blue light, sporulation depends only on the quality of the last irradiation. The sequence ending with near UV results in promotion of sporulation, and that ending with blue light results in inhibition of sporulation. The pigment system involved in this regulation has been described (Kumagai et al. 1976, Kumagai 1978). Fungi belonging to this group are Alternaria tomato (Kumagai 1983a), Helminthosporium oryzae (Honda et al. 1968, Yamamura et al. 1978, Kumagai 1983a), Botrytis cinerea (Tan 1974, Suzuki et al. 1977) and A. chichorii (Vakalounakis and Christias 1981, Kumagai 1983b).

Sporulation in Verticillium has been studied by Leach (1962), Osman and Valadon (1979) and Kaiser (1964) using 3- to 5-day-old colonies under continuous irradiation.

Both near UV and blue light were found to enhance sporulation. Our strain of V. agaricinum does not differ from other strains or species of Verticillium in that such radiation has a positive effect on sporulation. However, by adopting a different inoculation procedure, blue light was found to inhibit sporulation and UV light partially reverses the inhibitory effect of blue light, indicating the possible involvement of a UV and blue light photoreversible control mechanism similar to that described by Kumagai (1978). Paper IV and V deal mainly with the effect of culture conditions on photosporulation in V. agaricinum.

A survey of the results and interpretations presented in I-V is given in the following. In addition some original data (Figs. 2, 3 and 4), not previously published, are included in appropriate sections.

Abbreviations: ER, endoplasmic reticulum; UV, ultraviolet radiation (<400 nm); near UV (300-400 nm).

3 Light-induced carotenogenesis and in vivo absorbance changes in Verticillium agaricinum

The action spectrum for photocarotenogenesis has maxima at 290 nm (main peak) and 390 nm, and a minor peak at 483 nm (Fig. 1b). Light of wavelengths longer than 550 nm showed little activity. Under continuous irradiation for one day with light of these wavelengths, however, a significant amount of carotenoids accumulated as judged by the colour of the mycelial pads. Red light exposure of duration from 5 min to 1 h was ineffective in inducing carotenogenesis, consequently no phytochrome effect could be established (Tab. 2, I). Instead an interaction between UV and blue light was detected. Blue light partly reversed the UV induction of carotenogenesis when given after UV, but had no effect when given before UV. This gave a preliminary indication that the mycochrome system (Kumagai et al. 1976) or a similar pigment system could be involved.

A broad light-induced absorbance decrease centered at 384 nm was elicited most effectively by near UV (Fig. 1, II). It seems that a single pigment was involved in the changes since the maximum of the absorbance decrease was consistently at 384 nm irrespective of the duration of actinic exposure. The action spectrum (Fig. 2, II) for the absorbance changes has a maximum at about 380 nm, which corresponds quite well to the trough in the absorbance difference spectrum (Fig. 4, II).

The cell free preparation showed the same light-

induced absorbance changes when irradiated with 380 nm radiation whether alone or in the presence of potassium iodide (0.1 M) or sodium azide (5 mM). The chemicals had no effect on the amplitudes. This indicates that flavin is not involved (cf. Song and Moore 1968, Schmidt and Butler 1976). Neither did sodium dithionite (5 mM) nor potassium cyanide (5 mM) have any effect on the absorbance changes, while the respiration (measured as oxygen uptake by intact mycelia with a Clark electrode) was completely inhibited by 0.5 mM potassium cyanide. Thus the absorbance changes are independent of energy metabolism. It is most likely that the light-induced absorbance changes were due to photobleaching of a pigment with absorption maximum at about 380 nm, and that the bleaching has no connection with photocarotenogenesis. The same conclusion can be drawn by comparing the dark-minus-light difference spectrum with action spectra for light-induced absorbance changes and photocarotenogenesis (Fig. 4, II). There are practically no differences between the first two spectra. Both have a maximum at around 380 nm and both lack a secondary peak at 483 nm. In contrast, the action spectrum for photocarotenogenesis has a maximum at 390 nm and a secondary peak at 483 nm. There is a 25% difference in half peak width between the action spectrum for photocarotenogenesis and the other two spectra (Fig. 4, II). This also supports the conclusion that the light-induced absorbance changes and photocarotenogenesis are two independent processes.

4 Subcellular distribution of carotenoids in V. agaricinum

After disruption of the mycelia and upon further fractionation two major kinds of carotenoids were found. They are torulene (90%), found in the lipid layer on top of the supernatant, and an unidentified carotenoid (10%) associated with both mitochondria and endoplasmic reticulum at about 0.6 and 0.7-1.0 µg (mg protein)$^{-1}$, respectively (Tab. 3, III), equivalent to 1-2 carotenoid molecules per 1000 lipid molecules. Similar levels of membrane-associated carotenoids have been reported for ER (Mitzka-Schnabel and Rau 1980) and for mitochondria (Ramadan-Talib and Prebble 1978) in N. crassa. This suggests that there is a limited capacity for accomodation of carotenoids in ER and mitochondrial membranes and this capacity seems to be independent of the total content of carotenoids: 0.8 and 0.3 µg (mg protein)$^{-1}$ for V. agaricinum and N. crassa (Mitzka-Schnabel and Rau 1980), respectively.

The respiration of light-grown hyphae was found to be more susceptible to thermo-inactivation than the respiration of the dark control (unpublished results) (Fig. 2). In Acholeplasma laidlawii the fluidity of membranes decreases upon incorporation of carotenoids (Huang and Haug 1974). It is not known if the incorporation of carotenoids into membrane structure of V. agaricinum will have a similar effect on its fluidity or how this change will affect the thermo-stability of

its respiratory system. However, if the difference in thermo-stability between light- and dark-grown culture were caused by the incorporation of carotenoids, the pigment would be expected to be concentrated in special domains in order to exert any effect.

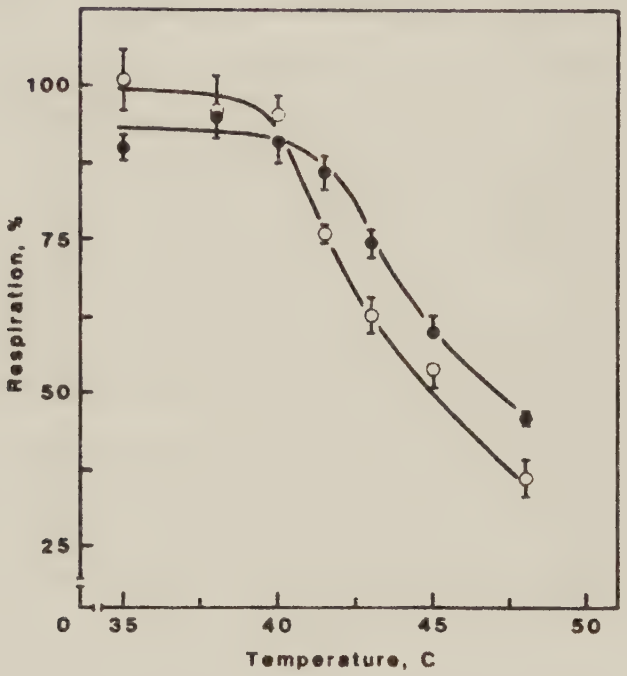

Fig. 2. The thermo-stability of V. agaricinum grown in the light and dark was investigated by subjecting the mycelial pads to a 10 min heat shock at different temperatures immediately prior to measurement of their respiration rate.

A circular nylon net (3.5 cm, diameter) was laid on top of the agar medium in the Petri dish at the time of inoculation. The mycelial pad was then peeled off in one single piece and attached to the water-jacketed roof of the oxygen electrode (Hansatech, Ltd., Norfolk, England) and the respiration rate of the fungus was measured. Respiration was expressed as % of oxygen consumption of the mycelium before heat shock treatment. Bars indicate the standard deviations of the mean of readings from four separate experiments.

Carotenoids, being widely distributed in all higher plants and microorganisms are the most extensive class of

compounds which are known to protect cells against photooxidation. In chloroplasts carotenoids are present in the thylakoid membrane where they serve, among other functions, to protect chlorophyll (Cogdell 1978). Carotenoids play a photoprotective role by efficiently quenching triplet exited photosensitizers and singlet oxygen (Krinsky 1978). Ramadan-Talib and Prebble (1978) observed a protective effect of carotenoids against photoinactivation of quinones in mitochondria of N. crassa. This could well be the physiological significance of photocarotenogenesis in V. agaricinum. Torulene which is not membrane associated, hence its absence from the sites of photodynamic action on the membranes, could still play a protective role by reducing the intensity of incoming visible light which reaches the membranes if the hyphae were more than several layers in thickness under natural conditions.

In N. crassa most of the carotenogenic actvities were present in the membrane fraction containing plasma membranes and, in particular ER, where also most of the membrane-bound carotenoids are located (Mitzka-Schnabel and Rau 1981). The subcellular site of carotenoid biosynthesis in V. agaricinum has not been investigated. However, assuming the results of N. crassa are applicable to V. agaricinum, this would mean that the majority of the carotenoids are synthesized in ER and then transported to mitochondria or to the cytosal after being converted to torulene.

5 Effect of growth conditions on the pattern of photosporulation in Verticillium agaricinum

Colonies of V. agaricinum produce very few conidia in the dark. A brief exposure to light followed by 2 days of darkness promotes sporulation to a level much higher than that obtained under continuous irradiation. Three types of broad band light sources, UV_{313}, near UV and blue light with emission peaks at 313, 355 and 450 nm, respectively, were tested. UV_{313} and near UV are both more effective in promoting sporulation than blue light (Fig. 6a, IV). A comparison of the effectiveness of the three broad band light sources for photosporulation (Fig. 6a, IV) and photocarotenogenesis (Fig. 6b, IV) show a striking similarity. UV_{313} and near UV are equally effective in initiating carotenogenesis (Fig. 6b, VI), while the action spectrum (Fig. 1d) suggests that UV_{313} should be more effective than near UV. The reason for this discrepancy is not clear.

With prolonged irradiation with UV_{313}, the colour of the colonies was changed from yellow to reddish, and this change was accompanied by growth retardation after 2 days of dark incubation. A similar colour change was observed when the dark grown colony was covered with NaOH. Thus the effect caused by irradiation with UV_{313} is similar to that caused by raising the pH. Spectra of absorbance changes induced by blue light irradiation and pH-induced absorbance changes are similar (Fig. 4). This suggests that light could cause a small pH change in the hyphae,

and, since the light-induced absorbance change is immediate, it could be the consequence of one of the early events in the reaction chain leading to sporulation.

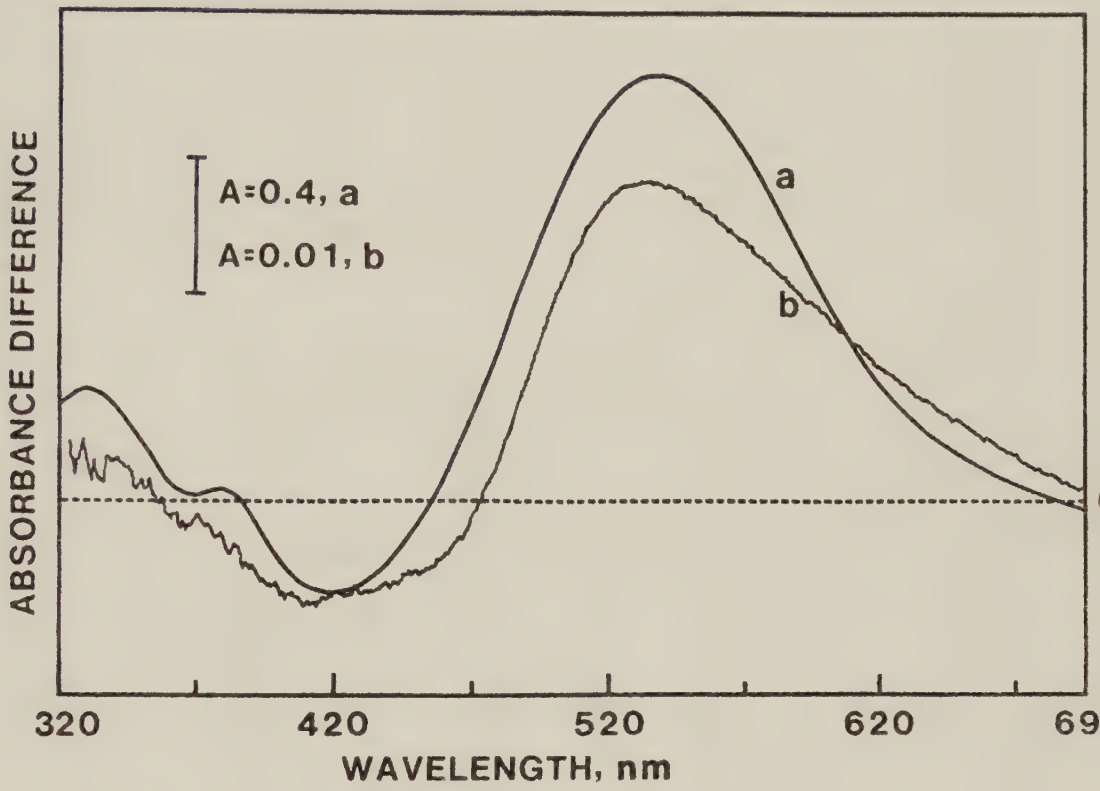

Fig. 3. Difference spectra of mycelial pad of V. agaricinum immediately before and after adding NaOH (a) and irradiating with light of 450 nm wavelength (b). One and a half ml conidial suspension (2 x 10^5 conidia ml^{-1}) in sucrose-yeast extract medium (Brandt and Reese 1964) at pH 6.0 was spread evenly over a Petri dish (5 cm, diameter) containing 10 ml of the medium with 2% agar. A 3-day-old dark grown mycelial pad was cut in strips and peeled off from the agar plate. After thorough washing with distilled water, a single strip was positioned between two opaque plastic plates, each with a hole much larger then the cross section of the measuring beam. The plates with the strip were placed in a 1 x 1 cm cuvette diagonally and irradiated with actinic light (150 W xenon lamp plus a Bausch & Lomb grating monochromator with variable slits fully open) at right angles to the measuring beam. The absorbance difference spectra were calculated with a Midan™ T Analyser (American Instrument Company) in line with the spectrophotometer (Aminco DW-2a).

When growth was started by inoculating with conidial suspension spread over the plates both near UV and blue light inhibited sporulation (Fig. 4). That the maximal inhibition was less than 80% was due to poor synchronization of the culture, since the conidia were not uniform in size and shape, and it takes about 5 h for a conidial population to achieve 100% germination. With continuous blue light irradiation, however, a total inhibition was obtained (Fig. 2, V).

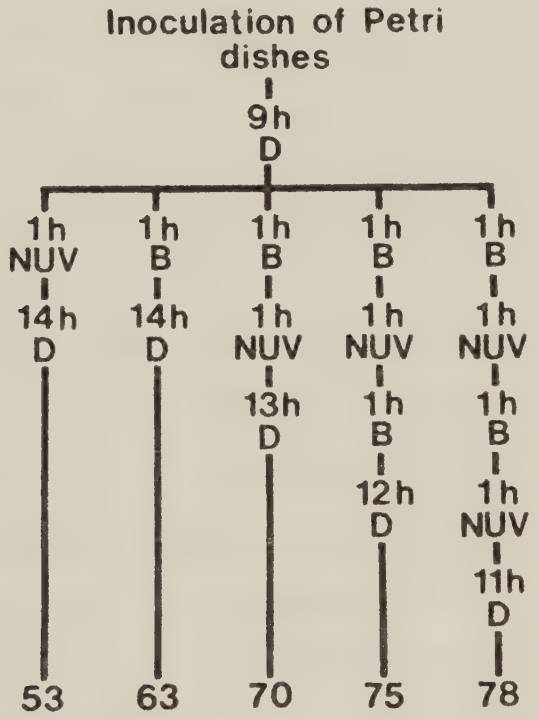

Fig. 4. Irradiation schedule for near UV (NUV) and blue (B) light. Exposures were carried out 9 h after inoculation when the young hyphae were in their most sensitive phase. Fluence rates were 0.8 and 1.0 W m^{-1} for blue and NUV light, respectively. Numbers (average of two plates) in the figure indicate % inhibition of sporulation. Both NUV and blue light have an inhibitory effect and there is no detectable NUV and blue photoreversible control of sporulation. The experiment was carried out at 20 °C.

A 10 s blue light pulse of 0.85 W m^{-2} saturated the response (Fig. 4,V). The sensitivity is comparable to induction by monochromic irradiation in A. tomato and H. oryzae (Kumagai 1983a). The inhibitory effect of blue light can be reversed to some extent by a subsequent pulse of UV_{313}. However, a further blue light pulse given after the UV_{313} light pulse has no effect. This is also true when a UV_{313} light pulse was given first in a sequence. It seems that the inhibitory effect of the blue light can be found only if it is given alone (Tab. 1, V). In certain fungi the effectiveness of blue light is temperature dependent (Leach 1967, Vakalounakis and Malathrakis 1983), being less effective at lower temperature. The absence of reversibility at 20 °C found in this fungus does not preclude its existence at a higher temperature.

The results show that near UV and blue light can have both stimulatory and inhibitory effects on sporulation depending on the growth condition of the fungus. It was found that potassium phosphate (KH_2PO_4 and K_2HPO_4 in the molar ratio of 9 to 7) in excess of 0.1 mM suppressed sporulation irrespective of light condition in colonies of V. agaricinum (Fig. 2 & 4, VI). In contrast potassium phosphate (KH_2PO_4, 18 mM and K_2HPO_4 14 mM) was necessary for dark sporulation when growth was started by inoculating with conidial suspension (Tab. 2, VI).

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7 References

Brandt, W. H. & Reese, J. E. 1964. Morphogenesis in Verticillium: A self-produced, diffusible morphogenetic factor. - Am. J. Bot. 51: 922-927.

Butler, W. L., Norris, K. H., Siegelman H. W. & Hendricks, S. B. 1959. Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. - Proc. Natl. Acad. Sci. USA 45: 1703-1708.

Cogdell, R. J. 1978. Carotenoids in photosynthesis. - Philos. Trans. R. Soc. London B284: 569-579.

De Fabo, E. C., Harding, R. W. & Shropshire, W., Jr. 1976. Action spectrum between 260 and 800 nanometers for photoinduction of carotenoid biosynthesis in Neurospora crassa. - Plant Physiol. 57: 440-445.

Gressel, J. B. & Hartmann, K. M. 1968. Morphogenesis in Trichoderma: Action spectrum of photoinduced sporulation. - Planta 79: 271-274.

Honda, Y., Sakamoto, M. & Oda, Y. 1968. Blue and near ultraviolet reversible photoreaction in the sporulation of Helminthosporium oryzae. - Plant Cell Physiol. 9: 603-607.

Huang, L. & Haug, A. 1974. Regulation of membrane lipid fluidity in Acholeplasma laidlawii: Effect of carotenoid pigment content. - Biochim. Biophys. Acta 352: 361-370.

Kaiser, W. J. 1964. Effect of light on growth and sporulation of Verticillium wilt fungus. - Phytopathology 54: 765-770.

Krinsky, N. I. 1978. Non-photosynthetic functions of

carotenoids. - Philos. Trans. R. Soc. London B284: 581-590.

Kumagai, T. 1978. Mychochrome system and conidial development in certain fungi imperfecti. - Photochem. Photobiol. 27: 371-379.

Kumagai, T. 1983a. Action spectra for the blue and near ultraviolet reversible photoreaction in the induction of fungal conidiation. - Physiol. Plant. 57: 468-471.

Kumagai, T. 1983b. Mycochrome system and the effect of molecular oxygen in the reversible photoinduction of conidiation in the fungus, Alternaria cichorri. - Physiol. Plant. 59: 590-594.

Kumagai, T. & Oda, Y. 1969. An action spectrum for photoinduced sporulation in the fungus Trichoderma viride. - Plant Cell Physiol. 10: 387-392.

Kumagai, T., Yoshioka, N. & Oda, Y. 1976. Further studies on the blue and near ultraviolet reversible photoreaction with an intracellular particulate fraction of the fungus, Alternaria tomato. - Biochim. Biophys. Acta 421: 133-140.

Leach, C. M. 1962. Sporulation of diverse species of fungi under near-ultraviolet radiation. - Can. J. Bot. 40: 151-161.

Leach, C. M. 1967. Interaction of near-ultraviolet light and temperature on sporulation of the fungi Alternaria, Cercosporella, Fusarium, Helminthosporium, and Stemphylium. - Can J. Bot. 45: 1999-2016.

Lipson, E. D. & Presti, D. 1977. Light-induced absorbance changes in Phycomyces photomutants. - Photochem. Photobiol. 25: 203-208.

Mitzka-Schnabel, U. & Rau, W. 1980. The subcellular distribution of carotenoids in Neurospora crassa. - Phytochemistry 19: 1409-1413.

Mitzka-Schnabel, U. & Rau, W. 1981. Subcellualr site of carotenoid biosynthesis in Neurospora crassa. - Phytochemistry 20: 63-69.

Muñoz, V. & Butler, W. L. 1975. Photoreceptor pigment for blue light in Neurospora crassa. Plant Physiol. 55: 421-426.

Neupert, W. & Ludwig, G. D. 1971. Sites of biosynthesis of outer and inner membrane porteins of Neurospora crassa mitochondria. - Eur. J. Biochem. 19: 523-532.

Osman, M. & Valadon, L. R. G. 1979. Effect of light quality on growth and sporulation in Verticillium agaricinum. - Trans. Br. Mycol. Soc. 72: 145-146.

Ramadan-Talib, Z. & Prebble, J. 1978. Photosensitivity of respiration in Neurospora mitochondria. A protective role for carotenoid. - Biochem. J. 176: 767-775.

Rau, W. 1967. Untersuchungen über die lichtabhängige Carotinoidsynthese I. Das Wirkungsspektrum von Fusarium aquaeductuum. - Planta 72: 14-28.

Rau, W. 1976. Photoregulation of carotenoid biosynthesis in plants. - Pure Appl. Chem. 47: 237-243.

Rily, G. J. P. & Bramley, P. M. 1976. The subcellular distribution of carotenoids in Phycomyces blakesleeanus C115 car-42 mad-107(-). - Biochim. Biophys. Acta 450: 429-440.

Schmidt, W. & Butler, W. L. 1976. Light-induced absorbance changes in cell-free extracts of Neurospora crassa. - Photochem. Photobiol. 24: 77-80.

Song, P. S. & Moore, T. A. 1968. Mechanism of the photo-dephosphorylation of menadiol diphosphate. A model for bioquantum conversion. - J. Am. Chem. Soc. 90: 6504-6514.

Suzuki, Y., Kumagai, T. & Oda, Y. 1977. Locus of blue and near ultraviolet reversible photoreaction in the stages of conidial development in Botrytis cinerea.- J. Gen. Microbiol. 98: 199-204.

Tan, K. K. 1974. Complete reversibility of sporulation by near ultraviolet and blue light in Botrytis cinerea. - Trans. Br. Mycol. Soc. 63: 203-205.

Tan, K. K. 1978. Light-induced fungal development. - In The Filamentous Fungi (J. E. Smith and D. R. Berry, eds.), pp. 334-357. Edward Arnold Publishers Ltd., London. ISBN 0-7131-2642-6.

Vakalounakis, D. J. & Christias, C. 1981. Sporulation in Alternaria cichorii is controlled by a blue and near ultraviolet reversible photoreaction. - Can. J. Bot. 59: 626-628.

Vakalounakis, D. J. & Malathrakis, N. E. 1983. Interaction of light quality and temperature on the vegetative reversion of conidiophores in Alternaria cichorii. - Can. J. Bot. 61: 626-630.

Valadon, L. R. G., Osman, M. & Mummery, R. S. 1979. Phytochrome mediated carotenoid synthesis in the fungus Verticillium agaricinum. - Photochem. Photobiol. 29: 605-607.

Valadon, L.R.G., Osman, M., Mummery, R. S., Jerebzoff-Quintin, S. & Jerebzoff, S. 1982. The effect of monochromatic radiation in the range 350 to 750 nm on the

carotenogenesis in Verticillium agaricinum. - Physiol. Plant. 56: 199-203.

Yamamura, S., Kumagai, T. & Oda, Y. 1978. Mycochrome system and conidial development in a nonphotoinduced isolate of Helminthosporium oryzae. - Can. J. Bot. 56: 206-208.

Zalokar, M. 1955. Biosynthesis of carotenoids in Neurospora. Action spectrum of photoactivation. - Arch. Biochem. Biophys. 56: 318-325.

The subcellular distribution of carotenoids in light-grown Verticillium agaricinum

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Hsiao, K.-C. and Møller, I. M. 1984. The subcellular distribution of carotenoids in light-grown Verticillium agaricinum. - Physiol. Plant. 00: 000-000.

Hyphae of light-grown V. agaricinum (Link) Corda containing many lipid bodies were disrupted by cutting and grinding in a buffer and membranes pelleted immediately by a 48 000 g spin for 30 min. The majority (90%) of the carotenoids were found in a lipid layer on top of the supernatant; its absorption maximum was at 488 nm in diethyl ether and on the basis of spectra in different organic solvents it was tentatively identified as torulene. The pellet contained 80% of the NAD^+-dependent malate dehydrogenase (marker for mitochondria), 25% of the antimycin-resistant NADH-cytochrome c reductase (marker for mitochondria and endoplasmic reticulum) and 10% of the proteins and the carotenoids. The latter were clearly different from the carotenoids in the lipid layer in that the absorption maximum was at 471 nm in diethyl ether.

Further fractionation of the 48 000 g pellet showed the presence of carotenoids in both

mitochondria and endoplasmic reticulum (ER) all with an absorption maximum at 471 nm. On a continuous Percoll gradient two main areas of mitochondrial activity (1.068 and 1.063 g ml^{-1}) were clearly separated from the ER (1.059 g ml^{-1}). The carotenoid content was about 0.6 and 0.7-1.0 μg (mg protein)$^{-1}$ for mitochondria and ER, respectively, equivalent to 1-2 carotenoid molecules per 1000 lipid molecules. The possible role of these membrane-bound carotenoid molecules is discussed.

Additional key words - Endoplasmic reticulum, fungus ,mitochondria, Percoll gradient, torulene.

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Introduction

In the cells of green plants carotenoids are present in the thylakoid membrane (Grumbach 1984). For fungi information on the subcellular distribution of this pigment is relatively scanty, particularly with respect to its possible membrane association. The majority of carotenoids in Phycomyces blakesleeanus was found in lipid globules suspended in the cytoplasm (Zalokar 1969,

Riley and Bramley 1976). In Blakeslea trispora, 6% of the bulk carotenoids were associated with membranes but the distribution of carotenoids among different membrane types as not investigated (Cederberg and Neujahr 1970). However, carotenoids were found in mitochondria from spores of P. blakesleeanus (Keyhani et al. 1972) as well as in mitochondria of Neurospora crassa (Ramadan-Talib and Prebble 1978). In the latter case the presence of carotenoids was shown to prevent photo-inactivation of the respiratory chain. As much as 23% of the total carotenoids were reported to be associated with the endoplasmic reticulum (ER) and very little with the mitochondria of N. crassa (Mitzka-Schnabel and Rau 1980). Thus, for N. crassa, which is the most well-studied organism, there is contradictory evidence on the association of carotenoids with the mitochondria. Riley and Bramley (1976) even suggested that carotenoid association with ER or mitochondria is an isolation artefact as they could detect no carotenoids in these membranes after purification from Phycomyces blakesleeanus.

Carotenogenesis in a number of fungi is under strict photo-control (Rau 1976). To these species belong Verticillium agaricinum (Hsiao and Björn 1982, Valadon et al. 1982). In V. agaricinum, the respiration of light-grown mycelia is more sensitive to thermo-inactivation than the respiration of dark-grown mycelia (K.-C. Hsiao , unpublished observation). Is this difference caused by the incorporation of carotenoids into the mitochondrial

membranes of light-grown cells? Is photo-carotenogenesis relevant for the survival of fungi under natural fluctuation of temperature and the spectral composition or intensity of light? We can only begin to answer these questions when information concerning the intracellular location of the carotenoids is available. In this contribution we present results on the subcellular distribution of carotenoids in V. agaricinum.

Abbreviations - BSA, bovine serum albumin; cyt c, cytochrome c; ER, endoplasmic reticulum; MDH, malate dehydrogenase; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,-tetra-acetic acid; TES, 2-(2-hydroxy-1,1-bis (hydroxymethyl)ethyl)aminoethanesulphonic acid.

Materials and methods

Organism and growth conditions

Verticillium agaricinum (link) Corda (ATCC 24668 = CMI 123870 from the American Type Culture Collection) was cultured as described by Hsiao and Björn (1982) with the exception that cellophane films were laid on top of agar medium in Petri dishes (9 cm diameter) before 4.8 ml of conidial suspension was spread over each dish. Cultures were kept in darkness for 24 h before exposure to 48 h continuous irradiation provided by fluorescence tubes (General Electric F48PG17 CWX Power Groove) at an intensity of 35 W m^{-2}. Mycelial pads, which had developed

an intense red colour, were then peeled off from the Petri dishes and washed with quartz-distilled water.

Electron microscopy

Mycelial pad was fixed in 2% (v/v) glutaraldehyde, and post-fixed in 2% (w/v) osmium tetroxide. After washing in 0.05 M cacodylate buffer, the hyphae were dehydrated in a graded series of ethyl alcohol and propylene oxide. The material was then embedded in Spurr's media (Spurr 1969). Section were cut with a diamond knife using a LKB V ultramicrotome, and thereafter stained with uranyl acetate and lead citrate.

Cell fractionation and density gradient centrifugation

A procedure modified from that of Schwitzguébel et al. (1981) was employed. Mycelial pads were squeezed dry between filter paper and weighed. The yield was about 0.4 g fresh weight $dish^{-1}$. Mycelia were then moistened with a small volume of isolation medium containing 0.35 M sucrose, 1 mM EGTA, 0.3% (w/v) BSA and 10 mM K^+-TES at pH 7.0. They were cut, 5 g at a time, for 2 min on a Teflon board with a rolling mincer (9 blades), and homogenized for 1 min in a mortar. The slurry was filtered through a layer of nylon net (66.14 x 79.53 mesh cm^{-1}), and the resulting suspension (called the homogenate) centrifuged at 48 000 g for 30 min in a Beckman J2-21M centrifuge. The pellet (designated P_{48}) was resuspended in wash medium (isolation medium without EGTA) and was further fractionated according to the scheme in Fig. 1. P_{10} and P_{30} were combined to give a total volume of 2.7 ml and

was applied on top of 40 ml wash medium containing 15% (v/v) Percoll (Pharmacia, Uppsala, Sweden). The centrifugation was carried out at 48 000 g for 1 h in a fixed angle rotor (JA-20). After centrifugation the gradient was collected by pumping with a peristaltic pump (1.3 ml min^{-1}) from the bottom of the tube and was monitored through an ultraviolet analyser (UVICORD, type 8301A, LKB Stockholm-Bromma, Sweden). Fractions of 1.3 ml were collected. All steps were carried out at 0-4°C under dim yellow light.

The density in the gradient fractions was calculated from the refractive index measured on a Zeiss refractometer at 21-22°C using the standard curve in the Percoll manual (Pharmacia 1980) and corrected for the difference in sucrose concentration.

Measurement of enzyme activities

Malate dehydrogenase (NAD^+-dependent, EC 1.1.1.37) was assayed in a medium containing 20 mM potassium 4-morpholinepropanesulphonate , pH 7.2, 0.36 μM antimycin A, 0.02% (v/v) Triton X-100, and 2 mM oxaloacetate. The reaction was started by the addition of 0.2 mM NADH and was followed at 340 nm. Antimycin A-insensitive NADH-cytochrome c reductase was assayed in a medium containing 50 mM K^+-TES, pH 7.5, 0.36 μM antimycin A, 1 mM KCN, 80 μM cyt c (oxidized). The reaction was started by the addition of 0.2 mM NADH and it was followed by the absorbance difference at 550-540 nm. Isocitrate lyase (EC 4.1.3.1.) was assayed as described by Schwitzguébel et

al. (1981) by following the formation of glyoxylate-phenylhydrazone at 324 nm.

Protein determination

Protein was measured by the method of Lowry et al. (1951) after solubilizing the membranes with 0.5% (w/v) deoxycholate. Bovine serum albumin (Fraction V ,96-99% albumin; Sigma Chemical Company) was used as the standard.

Determination of carotenoid content

Carotenoids were extracted with methanol and partitioned into diethyl ether or petroleum ether (Davies 1976). For measurements of absorption spectra in different solvents, the petroleum ether extract was evaporated at 35°C under N_2, and the residue was redissolved in the appropriate solvent. For carotenoids in the lipid layer, this layer was collected with a metal spatula and kept under N_2 in a desiccator. Carotenoids were then dissolved directly in different solvents. Absorption spectra and their 4th derivatives were measured with an Aminco DW-2a spectrophotometer in line with a Midan™ T Analyzer (American Instrument Company, Silver Spring, MD, USA). The amount of carotenoids was estimated from the spectrum using $E_{1\ cm}^{1\%} = 2500$ (Davies 1976) and the absorption maximum irrespective of its position. Wavelength calibration of the spectrophotometer was performed by using the deuterium emission line at 486 nm.

Results

Differential centrifugation

Preliminary experiments showed the presence of large amounts of lipids coloured orange-red by carotenoids in the homogenate of light-grown V. agaricinum. These lipids were mainly derived from the many lipid bodies found in the cells (Fig. 2). Since our aim was to investigate the possible presence of carotenoids in various membrane fractions in vivo, adsorption of carotenoids in vitro was to be avoided. To minimize membrane exposure to the carotenoid-lipid mixture, we therefore pelleted most of the membranes immediately with a 48 000 g spin for 30 min (Fig. 1). This yielded a red-coloured pellet (P_{48}) and a nearly colourless supernatant with a layer of orange-red lipids floating on the surface (S_{48}). P_{48} contained 10% of both the proteins and carotenoids present in the homogenate as well as 80% of the NAD^+-dependent malate dehydrogenase (MDH) and 25% of the antimycin-insensitive NADH-cyt c reductase (Tab. 1). NAD^+-dependent MDH is found in the mitochondrial matrix as well as in glyoxysomes and peroxisomes (Ting et al. 1975) if the latter are present. However, since isocitrate lyase, an enzyme characteristic of the glyoxylic acid cycle, was absent from the homogenate, P_{10} and P_{30} (activity <5 nmol (mg protein)$^{-1}$ min^{-1}) and the NADH-cyt c reductase is not found in peroxisomes, MDH in combination with NADH-cyt c reductase could be used as a convenient marker

for mitochondria. Antimycin A-insensitive NADH-cyt c reductase is found in the ER and the outer mitochondrial membrane (Moreau and Lance 1972). The specific activity of the mitochondrial outer membrane is, however, 3 times higher (Moreau and Lance 1972) and we therefore used the activity of NADH-cyt c reductase in combination with the absence of MDH as a marker for the ER. Resuspension of P_{48} and further fractionation by centrifugation (Fig. 1) yielded four fractions: P_1, presumably containing cell fragments and nuclei, P_{10} containing mitochondria, P_{30} with ER and some mitochondria (28% of the MDH) and S_{30} with ER (Tab. 1). There were carotenoids associated with all of these four fractions and the amount per mg protein varied only by a factor 3.6 (between P_1 and P_{30}).

Carotenoid spectra

It has been suggested that the presence of carotenoids in mitochondria and ER is an isolation artefact (Riley and Bramley 1976). We therefore compared the spectral properties of the carotenoids in the membrane fractions with those in the lipid layer (Fig. 3A). The carotenoids extracted from the lipid layer and representing 90% of the carotenoids had an absorption maximum at 488 nm in diethyl ether whereas carotenoids extracted from the combined P_{10} + P_{30} fraction had a peak at 471 nm. The 4th derivative spectra underlined the difference between carotenoids of the different fractions (Fig. 3B). They also show that the lipid layer, as well as the P_{10} + P_{30} fraction, each contained mainly one type of carotenoid

since there are no shoulders or extra peaks. The spectrum for carotenoids extracted from P_{48}, P_{10}, or P_{30} were essentially identical to that of $P_{10} + P_{30}$ shown in Fig. 3A (results not shown). The spectrum of carotenoids in P_1 (Fig. 3A) indicated that this fraction contained a mixture of carotenoids from the lipid layer and the membrane-bound carotenoids.

The absorption spectrum of intact hyphae showed a peak at 501 nm whereas the spectrum of P_{30} in buffer had a peak at 486 nm (Fig. 3C). In both cases, extraction of carotenoids into diethyl ether apparently shifted the maximum 13-15 nm towards lower wavelengths (compare Fig. 3A and 3C). P_{30} had an additional maximum at 416 nm (Fig. 3C) probably derived from heme-proteins present in the ER and the mitochondria.

Extraction of carotenoids into various solvents and comparing the position of the three main peaks on the 4th derivative spectra (see Fig. 3B) with values from the literature (Davies 1976) allowed us to make a tentative identification of the carotenoid in the lipid layer. This was probably torulene (Tab. 2) but thin-layer chromatography followed by gas-chromatography mass spectrometry is necessary for a definite identification. The membrane-bound carotenoid differed markedly in all solvents (Tab. 2) but we were unable to find values in the literature to fit the data.

Separation of membrane fractions on a Percoll gradient

The P_{30} fraction was clearly contaminated with mitochondria (Tab. 1) and a continuous self-generating

Percoll gradient was used to separate ER from the mitochondria. To increase the amount of mitochondria applied to the gradient and thereby facilitate detection we mixed P_{10} and P_{30} and separated the mixture on the gradient (Fig. 4). MDH showed the presence of mitochondria in three minor peaks at high density (fractions 7, 10 and 12) as well as a main peak in fraction 20. Above fraction 20, activity of MDH fell off sharply. NADH-cyt c reductase followed MDH closely up to fraction 21 as expected since both enzymes are found in the mitochondria, but showed high activity in fractions 23-28 where MDH was almost absent. Both proteins and carotenoids followed NADH-cyt reductase activity closely (Tab. 3).

The specific activities (per mg protein) of MDH and NADH-cyt c reductase in three peak fractions show a strong enrichment of NADH-cyt c reductase compared to MDH in fraction 27 (Tab. 3). This was also observed in another gradient (Tab. 3). Fractions 10 and 20 consist of mitochondria whereas fraction 27 contains ER and their densities differ significantly (Fig. 4, Tab. 3). The content of carotenoids (mg protein)$^{-1}$ varied little between the different membranes (Tab. 3) and the spectra of extracted carotenoids from the peaks (result not shown) were similar to that of $P_{10} + P_{30}$ shown in Fig. 3A.

Discussion

The membranes of light-grown V. agaricinum were removed from the contact with lipids and carotenoids present in the homogenate as quickly as possible. Free fatty acids are detrimemtal to the functional integrity of mitochondria (Wojtczak and Wojtczak 1960, Diolez and Moreau 1983) and as an extra precaution a high level of BSA (3 mg ml^{-1}) was used in all the media. Adsorption of carotenoids to the membranes during isolation should clearly also be avoided. It is not possible to prove that such adsorption did not take place, but we take the spectral difference between the membrane-bound carotenoids and the carotenoids found in the lipid layer (Fig. 3A for extracted carotenoids and Fig. 3C for carotenoids in situ) to be a good indication that these carotenoids are indeed found associated with the membranes in vivo. Keyhani et al. (1972) reported that the spectrum of isolated mitochondria from germinating spores showed the presence of carotenoids with a middle peak at 470 nm when measured in situ. Intact ungerminated spores, on the other hand, contained a carotenoid absorbing at 490 nm. This could indicate that in spores of Phycomyces there exists also two major types of carotenoids with different intracellular location.

The carotenoid in the lipid layer was tentatively identified as torulene (Tab. 2) which is acyclic at one

end and cyclic at the other. Torulene has also been been found as a major carotenoid in Rhodotorula minuta (Tada and Shiroishi 1982). The absorption maxima of the membrane-bound carotenoid were shifted to lower wavelengths by 7 to 20 nm compared with the carotenoid in the lipid layer. The absorption spectrum of the membrane-bound carotenoid does not have peaks as sharp as the spectrum for the carotenoid in the lipid layer, especially the lack of minimum between the main absorption peak and the shoulder at 443 nm (Fig. 3A). When the end group of an acyclic carotenoid undergoes ring closure, there is a displacement of the absorption maxima to shorter wavelengths, sometimes accompanied by a loss of persistence (Davies 1976). Furthermore, all the main carotenoids present in thylakoid membranes (Grumbach 1984) are cyclic at both ends. All this suggests that the membrane-bound carotenoid found in the present study could be cyclic, but we were unable to find published spectral peak values (Davies 1976) which matched the observed results (Tab. 2).

Hsiao and Björn (1982) reported that the absorption of carotenoids extracted from intact light-grown hyphae was 474 nm when measured in petroleum ether. This appeared at variance with the present results (486 nm for the lipid layer; Tab. 2) but the growth conditions differed slightly - Hsiao and Björn (1982) used 4-day old hyphae that had been exposed to light the final 24 h whereas we have here used 3-day-old cultures exposed to light for the final 48 h. When extracted with methanol, the former do indeed give an extract with a peak at 475 nm (result

not shown) in agreement with Hsiao and Björn (1982). However, when acetone was used as the extractant the peak was at 488 nm. It would therefore appear as if methanol only extracts part of the carotenoids (the membrane-associated?) from the cultures used by Hsiao and Björn (1982). In contrast to this, there was no difference between the spectra of methanol or acetone extracts obtained from the homogenate or from the $P_{10} + P_{30}$ fraction used in the present study (results not shown).

The carotenoids pelleted from the homogenate were associated with both the ER as well as with the mitochondria (Fig. 3, Tab. 1). We were careful to check that glyoxysomes were absent from our fractions since glyoxysomes on a Percoll gradient band at almost the same density as mitochondria in N. crassa (Schwitzguébel et al. 1981). The two areas of mitochondrial activity banded at 1.068 and 1.063 g ml^{-1} (Tab. 3) which is somewhat lower than the main peak of mitochondrial activity in N. crassa grown on sucrose (1.069-1.077 g ml^{-1}) or on acetate (1.078-1.089 g ml^{-1}; Schwitzguébel et al. 1981). These densities cannot be compared with results obtained using sucrose gradients because sucrose penetrates various organelles to different extent thereby changing their densities (see Schwitzguébel et al. 1981).

In fungi, carotenoids have been reported to be present in the mitochondria (Neupert and Ludwig 1970, Ramadan-Talib and Prebble 1978), in the ER but very little in the mitochondria (Mitzka-Schnabel and Rau 1980) or in some unidentified membrane fraction which was

neither mitochondria nor ER (Riley and Bramley 1976). Mitzka-Schnabel and Rau (1980) found only 0.02 µg carotenoids (mg protein)$^{-1}$ in the mitochondria compared to 0.85 µg mg^{-1} in the ER whereas we find almost the same amount of carotenoids (0.6-1.0 µg mg^{-1}) in the two membrane fractions (Tab. 3). Ramadan-Talib and Prebble (1978) found up to 0.6 µg carotenoids (mg protein)$^{-1}$ in the mitochondrial membranes which is in good agreement with our results (Tab. 3).

If one converts the amount of carotenoids from a protein basis (Tab. 3) to a lipid basis (assuming 1 mg protein equivalent to 1 mg lipids and an average molecular weight for lipids of 1000) the result is in the order of magnitude of between 1 and 2 carotenoid molecules per 1000 lipid molecules. In thylakoid membranes carotenoids are present at about 30 µg (mg protein)$^{-1}$ (Grumbach 1984) which works out at about 1 carotenoid molecule per 20 lipid molecules. Ramadan-Talib and Prebble (1978) observed a protective effect against photoinactivation of quinones in mitochondria of N. crassa at levels of carotenoids comparable to or lower than ours. It should not be overlooked that although the average concentration of carotenoids may be low in a given membrane, carotenoid molecules could be concentrated in specific domains thereby enhancing their effect. Protection against photoinactivation could be the physiological significance of carotenoid induction in V. agaricinum.

In V. agaricinum, the respiration of light-grown hyphae was more susceptible to thermo-inactivation then

the respiration of the dark-grown controls (K.-C. Hsiao, unpublished results). The rigidity of membranes increases upon incorporation of carotenoids (Huang and Haug 1974) provided the lipid composition is unchanged. It is possible that the incorporation of carotenoids into the mitochondrial membranes of light-grown hyphae could change the membrane properties and consequently the susceptibility of respiration to thermo-inactivation. Work is in progress to check this possibility.

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References

Cederberg, E. & Neujahr, H. 1970. Distribution of carotene in subcellular fractions of Blakeslea trispora. - Experientia 26: 366-367.

Davies, B. H. 1976. Carotenoids. - In Chemistry and Biochemistry of plant Pigments(T. W. Goodwin, ed), Vol. 2, pp. 38-165. Acadmic Press Inc. (London) Ltd. ISBN 0-12-289902-4.

Diolez, P. & Moreau, F. 1983. Effect of bovine serum albumin on membrane potential in plant mitochondria. - Physiol. Plant. 59: 177-182.

Grumbach, K. H. 1984. Does the chloroplast envelope contain carotenoids and quinones in vivo? - Physiol. Plant. 60: 180-186.

Hsiao, K.-C. & Björn, L. O. 1982. Aspects of photoinduction of carotenogenesis in the fungus Verticillium agaricinum. - Physiol. Plant. 54: 235-238.

Huang, L. & Haug, A. 1974. Regulation of membrane lipid fluidity in Acholeplasma laidlawii: Effect of carotenoid pigment content. - Biochim. Biophys. Acta 352: 361-370.

Keyhani, J., Keyhani, E. & Goodgal, S. H. 1972. Studies on the cytochrome content of Phycomyces spores during germination. - Eur. J. Biochem. 27: 527-534.

Lowry, O. H., Rosebrough, N. J. Farr, A. L. & Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. - J. Biol. Chem. 193: 265-275.

Mitzka-Schnabel, U. & Rau, W. 1980. The subcellular distribution of carotenoids in Neurospora crassa. - Phytochemistry 19: 1409-1413.

Moreau, F. & Lance, C. 1972. Isolement et proprietes des membranes externes et internes de mitochondries vegetales. - Biochimie 54: 1335-1348.

Neupert, W. & Ludwig, G. D. 1971. Sites of biosynthesis of out and inner membrane proteins of Neurospora crassa mitochondria. - Eur. J. Biochem. 19: 523-532.

Pharmacia Fine Chemicals AB. 1980. Percoll: Methodology and applications.

Ramadan-Talib, Z. & Prebble, J. 1978. Photosensitivity of respiration in Neurospora mitochondria. A protective role for carotenoid.- Biochem. J. 176: 767-775.

Rau, W. 1976. Photoregulation of carotenoid biosynthesis in plants. - Pure Appl. Chem. 47: 237-243.

Riley, G. J. P. & Bramley, P. M. 1976. The subcellular distribution of carotenoids in Phycomyces blakesleeanus C115 car-42 mad-107(-). - Biochim. Biophys. Acta 450: 429-440.

Schwitzguébel, J.- P., Møller, I. M. & Palmer, J. M. 1981. Changes in density of mitochondria and glyoxysomes from Neurospora crassa: A re-evaluation utilizing silica sol gradient centrifugation. - J. Gen. Microbiol. 126: 289-295.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. - J. Ultrastruct. Res. 26: 31-43.

Tada, M. & Shiroishi, M. 1982. Mechanism of photoregulated carotenogenesis in Rhodotorula minuta I. Photocontrol of carotenoid production. - Plant Cell Physiol. 23: 541-547.

Ting, I. P., Fuhr, I., Curry, R. & Zschoche W. C. 1975. Malate dehydrogenase isozymes in plant: Preparation, properties, and biological significance. - In Isozymes (E. L. Mackert, ed.) Vol. II, pp. 369-384. Academic Press, New York. ISBN 99-0329009-8.

Valadon, L. R. G.,Osman, M., Mummery, R. S., Jerebzoff-Quintin, S. & Jerebzoff, S. 1982. The effect of monochromatic radiation in the range 350 to 750 nm on the carotenogenesis in Verticillium agaricinum. - Physiol. Plant. 56: 199-203.

Wojtczak, L. & Wojtczak, A. B. 1960. Uncoupling of oxidative phosphorylation and inhibition of ATP-P_i exchange by a substance of insect mitochondria. - Biochim. Biophy. Acta 39: 277-286.

Zalokar, M. 1969. Intracellular centrifugal separation of organelles in Phycomyces. - J. Cell Biol. 41: 494-509.

Tab. 1. Distribution of proteins, carotenoids and activities of marker enzymes amongst subcellular fractions of light-grown V. agaricinum. Fractions were obtained by the method described in the text (see also Fig. 1). Data are given as mean ± SE (number of separate experiments).

Fraction	Protein	Carotenoid	MDH	NADH-cyt c reductase
	mg	µg	µmol min^{-1}	µmol min^{-1}
Homogenate	530 ±320(3)	421 ±75(3)	52 ± 24(3)	21 ± 14(2)
	% of homogenate			
P_{48}	10 ± 3(3)	10 ± 5(3)	80 ± 8(3)	26 ± 21(2)
S_{48}	98 ± 8 (3)	88 ± 7(3)	17 ± 2(3)	75 ± 8(2)
Subfractionation of P_{48}				
P_1	1.1 ± 0.4(3)	0.5 ± 0.2(3)	2.6 ± 0.9(3)	0.4 ± 0.2(2)
P_{10}	2.8 ± 1.4(3)	1.9 ± 0.8(3)	43 ± 12(3)	4.8 ± 1.3(2)
P_{30}	3.5 ± 1.4(3)	4.2 ± 1.0(3)	28 ± 3(3)	5.6 ± 4.0(2)
S_{30}	2.6 ± 1.4(3)	4.0 ± 0.1(2)	2.4 ± 0.9(3)	7.7 ± 5.4(2)

Tab. 2. Identification of two major carotenoids of V. agaricinum in different solvents. Membrane-bound carotenoid (fraction P_{10} and P_{30}) and carotenoid in the lipid layer (top layer of S_{48}) were extracted into various solvent and absorption maxima were read off from the 4th derivative spectra measured with an Aminco DW-2a spectrophotometer in line with a Midan™ T Analyzer. Values in parentheses are absorption maxima read directly from the absorption spectra. Absorption maxima for torulene were taken from tables compiled by Davies (1976).

	Measured								
Solvents	Membrane-bound			Lipid layer			Torulene		
Benzene	—	—	—	471	505 (504)	539	470	503	541
Carbon disulphide	—	—	—	492	527 (525)	563	491	525	563
Diethyl ether	443	471 (474)	507	459	488 (488)	520	—	—	—
Hexane	447	478 (479)	513	457	487 (487)	520	458	487	519
Petroleum ether	446	474 (475)	508	456	486 (484)	519	460	484	518
Pyridine	457	490 (491)	527	477	510 (508)	545	475	508	545

Tab. 3. Specific activities of marker enzymes, carotenoids and density of main peak fractions obtained from the Percoll density gradient centrifugation of membrane fraction (P_{10} + P_{30}) of light-grown V. agaricinum shown in Fig. 3. Units of enzyme activities are μmol (mg protein)$^{-1}$ min^{-1}. Carotenoids and densities are in μg (mg protein)$^{-1}$ and g ml^{-1}, respectively. Figures in parentheses are data from other gradient(s) of separate preparation(s).

	Fraction number		
Parameter	10	20	27
Density (average ± SE for three gradients)	1.068	1.063 (1.065 ± 0.002)	1.057 (1.059 ± 0.0015
Carotenoids (from another gradient)	0.60	0.60 (0.60)	0.72 (0.96)
MDH	1.5	1.7	0.24
NADH-cyt c red	0.11	0.22	0.15
MDH/NADH-cyt c red (from another gradient)	14.4	7.7 (9.5)	1.6 (0.8)
Identity of particles	mitochondria	mitochondria	endoplasmic reticulum

Figure legends

Fig. 1. Flow-sheet for cell fractionation.

Fig. 2. Electron micrograph of light-grown hyphae of Verticillium agaricinum showing many large lipid bodies (LB). The bar is 1.8 /um.

Fig. 3. Absorption spectra of two main types of carotenoids, membrane-bound and in lipid layer, found in light-grown V. agaricinum. (A), absorption spectra of diethyl ether extract of lipid layer (a), P_{10} +P_{30} (b) and P_1 (c); (B), the corresponding 4th derivative spectra of a and b (d and e, respectively); and (C), absorption spectra of intact hyphae (f) as well as P_{30} suspended in wash medium (g). Numbers in B indicate the position of the main absorption maxima of carotenoids. Absorption spectrum of intact hyphae (f) represents the in vivo absorption of the carotenoids found in lipid layer. Absorption spectrum of P_{30} suspended in wash medium represents the in situ absorption of membrane-bound carotenoid (g). The additional maximum at 416 nm in g probably derived from heme-protein in ER and mitochondria. Absorbance is not indicated in this figure. Spectra a and b, and f and g are normalized.

Fig. 4. Distribution of enzyme activities, carotenoids and protein after separation of P_{10} + P_{30} mixture on a self-generating continuous Percoll

gradient. The gradient, 15% Percoll (v/v), was centrifuged at 48 000 g for 1 h in a fixed angle rotor. MDH activity is given as μmol min^{-1} $fraction^{-1}$, NADH-cyt c reductase as nmol min^{-1} $fraction^{-1}$, protein as mg ml^{-1} and carotenoids as A_{471} $fraction^{-1}$.

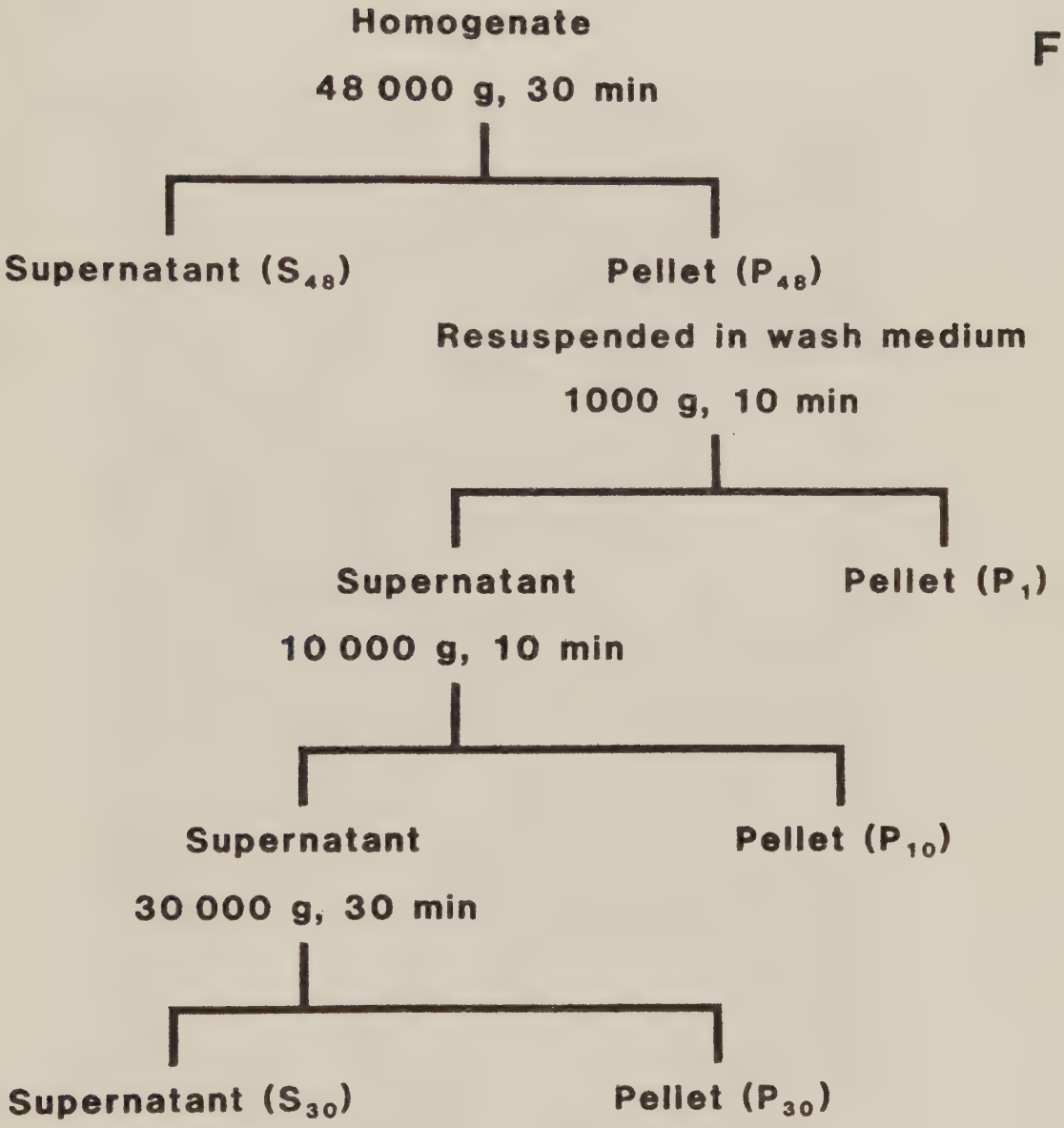

Homogenate
48 000 g, 30 min
Supernatant (S_{48})
Pellet (P_{48})
Resuspended in wash medium
1000 g, 10 min
Supernatant
Pellet (P_1)
10 000 g, 10 min
Supernatant
Pellet (P_{10})
30 000 g, 30 min
Supernatant (S_{30})
Pellet (P_{30})

Fig. 1

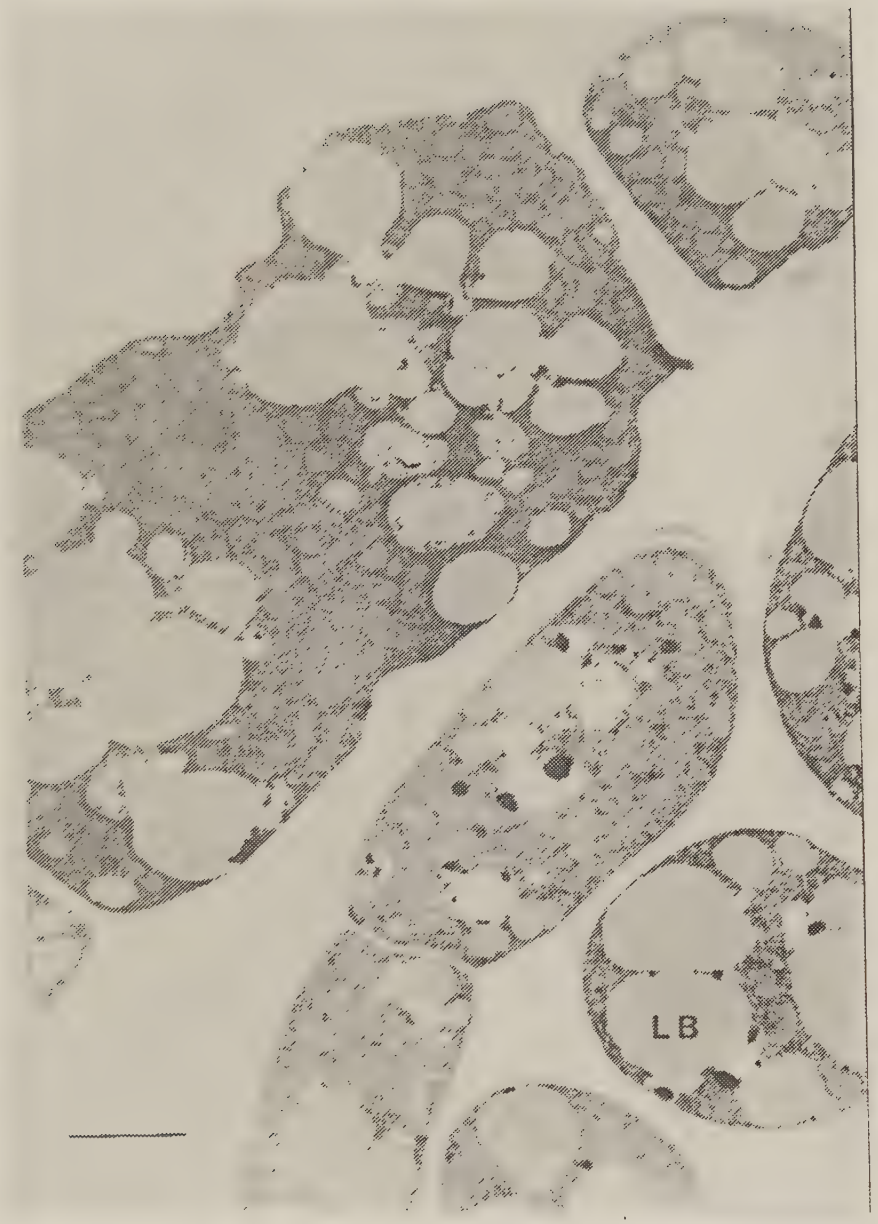


Fig. 2

Fig. 3

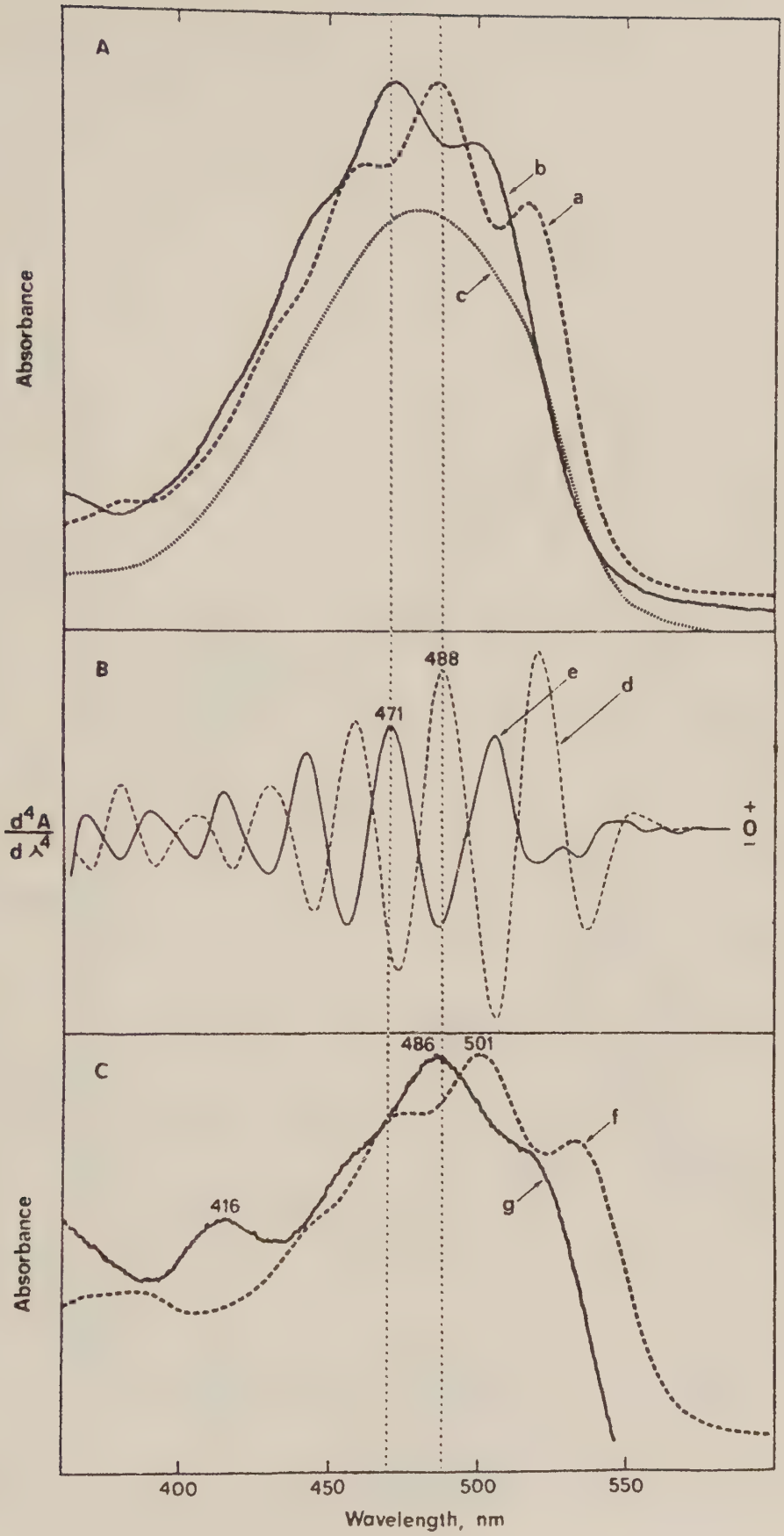
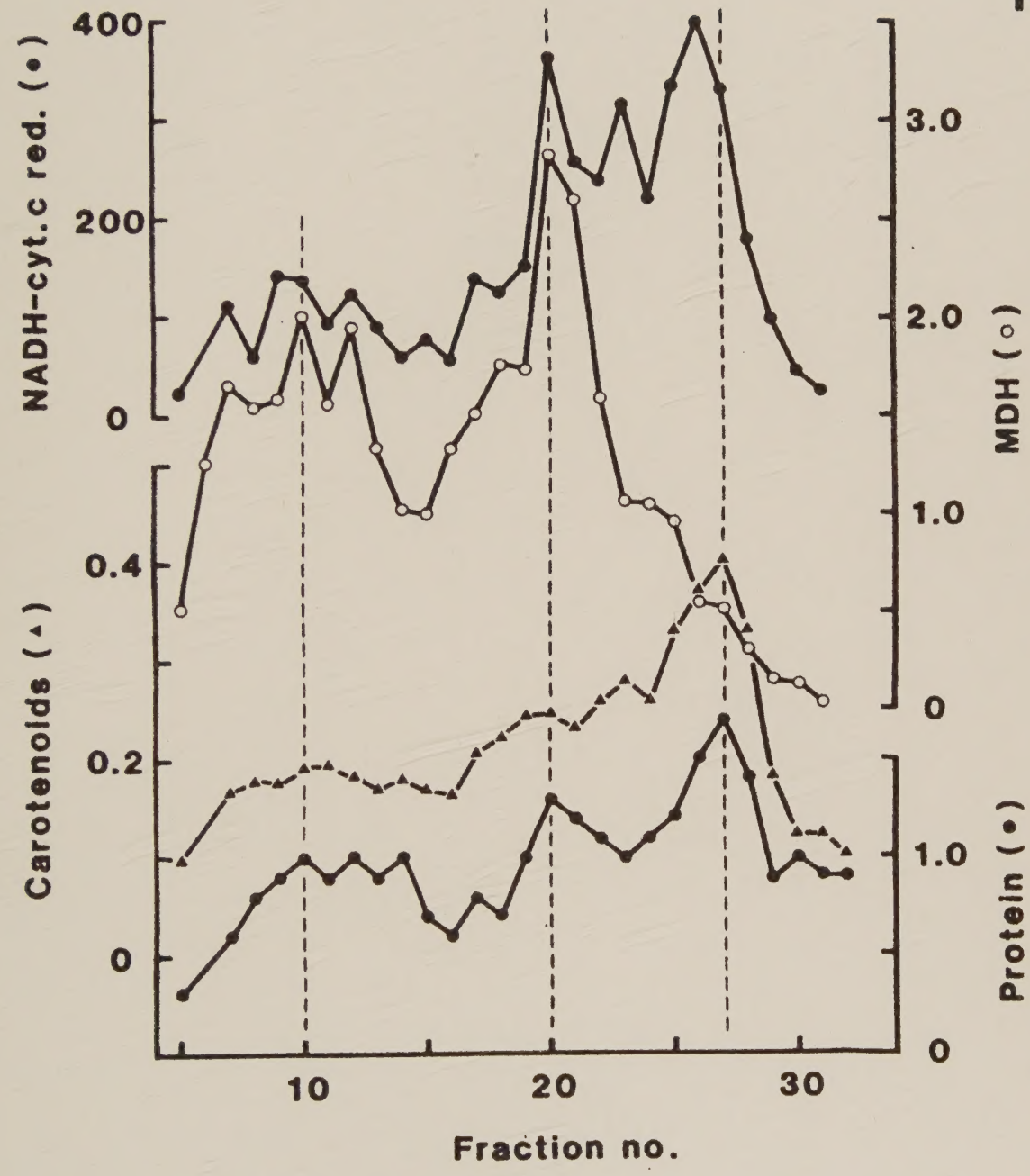

A
Absorbance
b
a
c
B
488
471
e
d
$\frac{d^4A}{d\lambda^4}$
+
0
−
C
486
501
f
416
g
Absorbance
400
450
500
550
Wavelength, nm

Fig. 4


NADH-cyt.c red. (•)
400
200
0
MDH (○)
3.0
2.0
1.0
0
Carotenoids (▲)
0.4
0.2
0
Protein (•)
1.0
0
10
20
30
Fraction no.

3.0
2.0
1.0
0
NDH (○)
1.0
0
Protein (●)
30
20
10
Fraction no.
400
200
0
NAD(H)-CN-sensitive (○)
0.4
0.2
0
Carotenoids (△)